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**14. ABSTRACT** We have analyzed the effect of a CXCR4 antagonist, expressed by oncolytic vaccinia virus or delivered in a soluble form by an intravenous or intraperitoneal injection, on metastatic dissemination of an invasive variant of the murine epithelial ovarian cancer cell line ID8-T. Using an orthotopic ID8-T tumor model in syngeneic mice, we have demonstrated that intraperitoneal delivery of a CXCR4 antagonist-expressing virus was more efficacious against ID8-T tumor than a systemic injection of the armed vector or soluble antagonists. The inhibition of tumor growth was dependent on the intratumoral concentration of the CXCR4 antagonist and virally-induced inflammation characterized by infiltration of CD8+ T cells and IL-12 cytokine-expressing tumor associated macrophages with concomitant inhibition of the immunosuppressive network. We conclude that targeting the CXCR4 signaling though intraperitoneally delivered oncolytic virotherapy together with the CXCR4 antagonist yields a significant therapeutic impact against metastatic ovarian cancer and may be included in multimodal virotherapy-based treatments in combination with cancer vaccines and adoptively-transferred T cells.

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## 1. INTRODUCTION:

Although it is well established that the CXCR4 receptor for the CXCL12 chemokine forms a pivotal signaling pathway that enables metastasis by epithelial ovarian carcinomas, a larger gap remains in our understanding of the mechanism that governs the impact of modulation of the CXCL12/CXCR4 axis on inhibition of tumor growth and induction of antitumor immune responses. Several soluble CXCR4 antagonists have demonstrated antitumor efficacy in preclinical models and have been evaluated in clinical trials. However, while the disruption of the CXCL12/CXCR4 pathway inhibits tumor growth and metastatic spread, it can also affect the development and efficacy of antitumor immune responses because of expression of CXCR4 on several subsets of immune cells. Therefore, a delivery mode of the CXCR4 antagonist (soluble or tumor-targeted through expression by an oncolytic virus) can be an important factor in modulating the induction, migration, and function of tumor-specific T cells due to differences in concentration of the CXCR4 antagonist in the tumor versus systemic tissues. In this study we compared the effects of targeted versus systemic delivery of the CXCR4 antagonist on inhibition of ovarian tumor growth, angiogenesis and immunosuppressive networks in the tumor microenvironment. The CXCR4 antagonist, expressed in the context of the murine Fc fragment of IgG2a (CXCR4-A-Fc), was delivered as a soluble protein or by oncolytic vaccinia virus (OVV-CXCR4-A-Fc) to syngeneic mice after orthotopic challenge with metastatic ID8-T ovarian tumor. We found that intraperitoneal delivery of OVV-CXCR-A-Fc was more efficacious in inhibiting tumor growth than the intravenous injection of the armed virus or treatment with the soluble antagonist. We have also determined that the intratumoral level of the CXCR4-A-Fc antagonist together with the virally-induced inflammation characterized by increased infiltration CD8$^+$ T cells, dendritic cells (DCs) and IL-12-expressing inflammatory macrophages influenced for the generation of antitumor responses. The following is a detailed account of our progress made for each task outlined in the original SOW.

## 2. KEY WORDS:

CXCR4 antagonist, Oncolytic vaccinia virus, Ovarian tumor, Tumor microenvironment, Immunosuppression, Immune responses, Pathogenicity

## 3. ACCOMPLISHMENTS:
**What were the major goals of the project?**

**Body (from Original SOW):** Specific Aim 1. We have compared inhibition of tumor growth and kinetics of recruitment of immunosuppressive cells to metastatic ovarian ID8-T tumors during treatment of tumor-bearing mice with soluble or tumor-targeted CXCR4 antagonist.

**Task 1.** C57BL/6 mice were challenged orthotopically with ID8-T syngeneic ovarian tumor cells as previously described (1). The tumor-bearing mice were treated with soluble CXCR4-A-Fc or virally-delivered OVV-CXCR4-A-Fc antagonist injected intravenously (i.v.) or intraperitoneally (i.p.) 10 days after tumor challenge. The soluble CXCR4 antagonist was delivered daily for 7 days to simulate a 1-wk production of the antagonist from OVV-CXCR4-A-Fc-infected tumor cells. As controls, additional groups of tumor-bearing mice received a clinically approved CXCR4-antagonist known as AMD3100-a bicyclam/plerixafor (AMD3100) or oncolytic vaccinia virus expressing the Fc portion of Ig (OVV-Fc). Because OVV can directly destroy tumor cells, to fairly compare the survival benefit of the OVV-delivered CXCR4 antagonist with that of the soluble counterpart delivered alone, we included an additional group of mice that received OVV-Fc plus soluble CXCR4-A-Fc antagonist. The concentration of the CXCR4-A-Fc antagonist after each treatment was monitored in sera, as well as tumor, liver, bone marrow (BM), lymph node, and spleen tissues by ELISA.

**Progress:**

1a. *Preparation of oncolytic vaccinia viruses and soluble CXCR4-A-Fc antagonists* (*timeframe, during months 1-12*): We have amplified OVV-CXCR4-A-Fc and OVV-Fc viruses on HuTK⁻ 143 fibroblasts followed by purification over a sucrose gradient, titration on monkey kidney fibroblasts CV1 for *in vivo* studies in ID8-T tumor-bearing syngeneic C57BL/6 mice. The soluble CXCR4 proteins, consisting of the CXCR4 antagonist expressed in the context of Fc fragment of murine IgG2a or human IgG1 with disulfide bonds in a hinge region for preservation of its dimeric structure, were collected in supernatants of HuTK⁻ 143 fibroblasts infected with the armed OVV at multiplicity of infection (MOI) of 1 for 24 hrs. After centrifugation, cell-free supernatants were filtered and the CXCR4 fusion proteins were purified on protein G column as described (2). The preparations of soluble CXCR4-A-Fc and the oncolytic vaccinia virus are conducted on a regular basis every 4 months.

1b. *Inhibition of ID8-T ovarian tumor growth* (*timeframe, during months 1-10*). We have compared antitumor efficacy of soluble and virally-delivered CXCR4-A-Fc fusion protein against an invasive variant of the murine epithelial ovarian cancer cell line ID8-T growing orthotopically in syngeneic C57/BL6 mice. The soluble CXCR4-A-Fc fusion protein (5 mg/kg) or virally-delivered OVV-CXCR4-A-Fc antagonist ($10^8$ PFU) were injected i.v. or i.p. to tumor-bearing mice 10 days after tumor challenge. The soluble CXCR4-A-Fc fusion protein was delivered daily for 7 days to simulate a 1-wk production of the antagonist from *in vivo* OVV-CXCR4-A-Fc-infected tumor cells. As controls, additional groups of tumor-bearing mice received AMD3100 antagonist (5 mg/kg), OVV-Fc or a combination of OVV-Fc and daily delivered soluble CXCR4-A-Fc fusion protein. Progression of tumor growth, quantified by bioluminescence imaging, revealed rapid tumor cell dissemination in control mice that were killed within 4 weeks after tumor challenge (Fig. 1A and B). Treatments with AMD3100 and the CXCR4-A-Fc fusion protein delivered either i.v. or i.p. had similar inhibitory effects on tumor growth ($P < 0.05$). The tumor burden after systemic delivery of OVV-CXCR4-A-Fc was significantly reduced compared with control and OVV-Fc-treated mice (Fig. 1A; $P = 0.02$ and $P = 0.05$, respectively), and more pronounced antitumor effects were detected after i.p. treatment with the armed and control viruses (Fig. 1B; $P = 0.01$ and $P = 0.04$, respectively). However, the combined treatment of OVV-Fc and soluble CXCR4-A-Fc fusion protein exhibited the highest antitumor effect and extended the dormancy period for approximately 5 weeks (Fig. 1A and B).
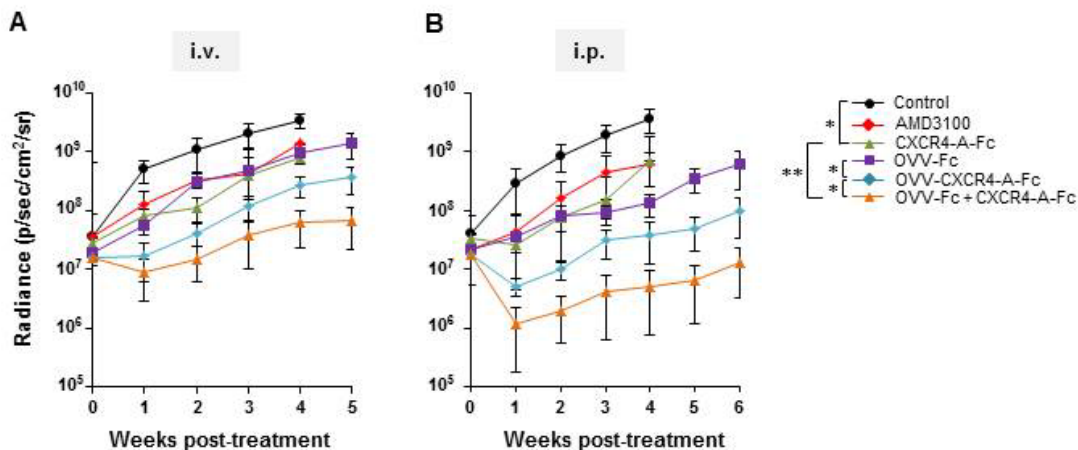


**Figure 1.** Antitumor activity of oncolytic virotherapy and CXCR4 antagonists against orthotopic ID8-T tumor in syngeneic mice. C57BL/6 female mice ($n = 8$) were challenged i.p. with ID8-T syngeneic ovarian tumor cells ($5\times10^5$ cells). The tumor-bearing mice were treated with soluble CXCR4-A-Fc (5 mg/kg), or virally-delivered OVV-CXCR4-A-Fc antagonist ($10^8$ PFU) injected i.v. (**A**) or i.p. (**B**) 10 days after tumor challenge. The soluble CXCR4 antagonist, purified from supernatant of OVV-CXCR4-A-Fc-infected HuTK⁻ fibroblasts on a Protein G column, was delivered daily for 7 days. Additional groups of tumor-bearing mice received AMD3100 (5 mg/kg) or OVV expressing the Fc portion of murine IgG2a (OVV-Fc). Control mice were treated with PBS. Tumor progression was monitored by bioluminescence imaging using the Xenogen IVIS Imaging System. Data points represent mean ± SD. *$P < 0.05$, **$P < 0.01$.

1c. *Accumulation of the CXCR4-A-Fc antagonist in tumor tissues, sera and lymphoid organs after viral delivery versus injection of the soluble fusion protein* (*timeframe, during months 1-10*): The finding of higher antitumor efficacy achieved with the combined treatment of OVV-Fc and soluble CXCR4-A-Fc antagonist compared to the efficacy of each treatment alone, raises questions of the role of intratumoral accumulation of the antagonist in eliminating malignant cells through modification of the inflammatory responses induced by the virus in the tumor microenvironment. To address this concern, we next analyzed differences in intratumoral concentrations of the CXCR4-A-Fc antagonist after i.v. or i.p. delivery of OVV-CXCR4-A-Fc or injection of the soluble fusion protein for 7 days alone or in combination with OVV-Fc. For the analysis, we used the CXCR4-A-Fc fusion protein consisting of human instead of murine Fc fragment to avoid cross-reactivity with endogenous murine antibodies. The levels of the CXCR4-A-Fc protein in tumor tissues and different organs were analyzed on day 8 after each treatment by ELISA using cell lysates and normalized the values to total protein content. The results depicted in Fig. 2A and B show that the intratumoral concentrations of CXCR4-A-Fc protein released from virally-infected tumor cells after OVV-CXCR4-A-Fc treatment were over 2-fold lower compared to those achieved by 7-day injections of the soluble counterpart. The intratumoral injection of the soluble CXCR4-A-Fc protein resulted in approximately twofold higher levels of the antagonist in the tumor tissues compared to those accumulated after the i.v. delivery ($P = 0.05$). The highest levels of the CXCR4-A-Fc protein were detected in sera after i.v. treatment with the soluble antagonist, which were greater than those detected in tumor tissues of the same mice. Low accumulation (~40 pg/mg protein) of the CXCR4-A-Fc protein was also detected in lymph nodes, BM, liver and spleen after systemic delivery, whereas only a background level was found in these organs after the i.p. treatment. This analysis revealed that more selective accumulation of the CXCR4-A-Fc antagonist in tumor tissues after i.p. than i.v. delivery could contribute to the increased antitumor efficacy.
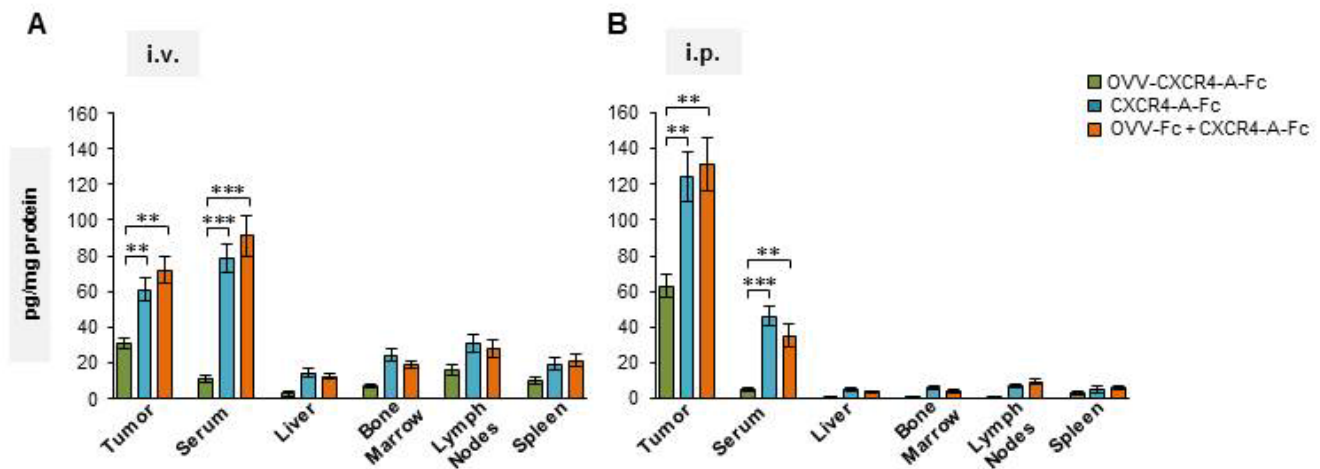


**Figure 2.** Accumulation of the CXCR4-A-Fc antagonist in tumor tissues, sera and lymphoid organs after intravenous (**A**) or intraperitoneal (**B**) delivery of OVV-CXCR4-A-Fc or injection of the soluble CXCR4-A-Fc fusion protein to ID8-T tumor-bearing mice. Concentrations of CXCR4-A-Fc fusion protein were determined on day 8 after the treatment by ELISA in sera, and cell lysates of tumor, liver, BM, lymph nodes (axillary, brachial, and inguinal), and spleen tissues after normalization to total protein content. Data are presented as the mean ± SD of five mice per group. **P < 0.01, ***P < 0.001.

**Task 2:** The levels of the CXCR4-A-Fc fusion protein in tumor tissues were correlated with changes in the tumor microenvironment.

**Progress:**

2a. *The effect of differences in concentrations of the CXCR4-A-Fc antagonist in tumor tissues after systemic and i.p. delivery on recruitment of CD8$^+$ T cells, inflammatory monocyte/macrophages, myeloid-derived suppressor cells (MDSCs), T regulatory cells (Tregs), and hematopoietic stem and progenitor cells (HSCs/HPCs)*

(*timeframe, during months 5-8*): The overall goal of this aim was to compare the induction of spontaneous antitumor immune responses and kinetics of recruitment of immunosuppressive cells in the tumor stroma by systemic and intraperitoneal modes of the CXCR4-A-Fc antagonist delivery. We anticipated that inhibition of peritoneal dissemination of ID8-T tumor after the armed oncolytic virotherapy treatment was mediated by a direct cytopathic effect of the virus on tumor cells and the induction of inflammatory responses by the virus due to the ability of vaccinia to break Treg-mediated tolerance (3) as well as inhibition of the immunosuppressive network in the tumor by the CXCR4-A-Fc antagonist. Consistent with this hypothesis, flow cytometry analysis of ascites-derived tumors or peritoneal washes obtained from the tumor-challenged mice on day 8 after the treatment revealed increased intratumoral infiltration of $CD8^+$ T cells and $CD11b^+Ly6C^+$ inflammatory monocytes/macrophages after oncolytic virotherapy compared to the untreated mice or animals injected with AMD3100 or soluble CXCR4-A-Fc protein (Fig. 3A and B). The inhibitory effect of the antagonists was more prevalent after i.p. than i.v. treatment.
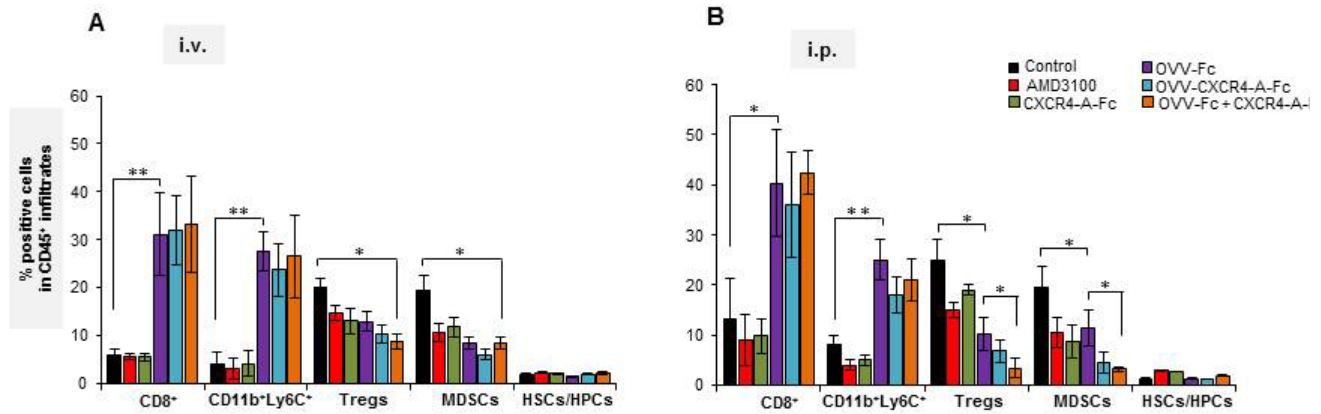


**Figure 3.** Evaluation of immune infiltrates in ascites-derived tumors or peritoneal washes by oncolytic virotherapy treatments alone or in combination with the CXCR4 antagonists delivered i.v. (**A**) or i.p. (**B**). Frequencies of $CD8^+$ T cells, $CD11b^+Ly6C^+$ inflammatory monocytes/macrophages, $CD4^+CD25^+Foxp3^+$ Tregs, $CD11b^+Ly6G^+$ MDSCs, and c-$Kit^+Lin^-Sca-1^+$ HSCs/HPCs in immune infiltrates were analyzed by flow cytometry 8 days after treatments. Results are presented as mean ± SD of five mice per group. *$P < 0.05$, **$P < 0.01$.

The reduced antitumor efficacy of OVV-Fc compared to that mediated by the armed virus or a combination of OVV-Fc and the CXCR4-A-Fc fusion protein, despite similar numbers of tumor-infiltrating $CD8^+$ lymphocytes, emphasized the need for blocking the CXCL12/CXCR4 signaling axis to increase inhibition of the immunosuppressive network in the tumor microenvironment. This observation was consistent with lower accumulation of $CD4^+CD25^+Foxp3^+$ Tregs in mice treated with the CXCR4-A-Fc antagonist or OVV-CXCR4-A-Fc compared to control animals or those receiving OVV-Fc therapy only ($P = 0.03$ and $P = 0.05$, respectively). A similar profile of treatment-mediated responses was measured for $CD11b^+Ly6G^+$ MDSCs. Altogether, results of this analysis indicate that delivery of the armed virus or a combination of virus with the soluble antagonist is able to alter the inflammatory status of the tumor microenvironment in favor of immune activity over immune suppression. It is also noteworthy that the highest levels of the CXCR4-A-Fc fusion protein in the tumor after i.p. treatment did not affect mobilization of HSCs/HPCs based on comparable numbers of these cells in untreated tumor-bearing mice and those receiving the antagonist.

Consistent with the flow cytometry analysis, histology performed on formalin-fixed and haematoxylin and eosin (H&E)-stained sections of omental tumor tissues obtained 30 days after each treatment revealed increases in infiltration of leukocytes after treatment with oncolytic vaccinia virus, and the changes were similar after intravenous and intraperitoneal injections (Fig. 4A and B). In contrast, tumor tissues from control mice or those treated with the soluble antagonist were poorly inflamed.
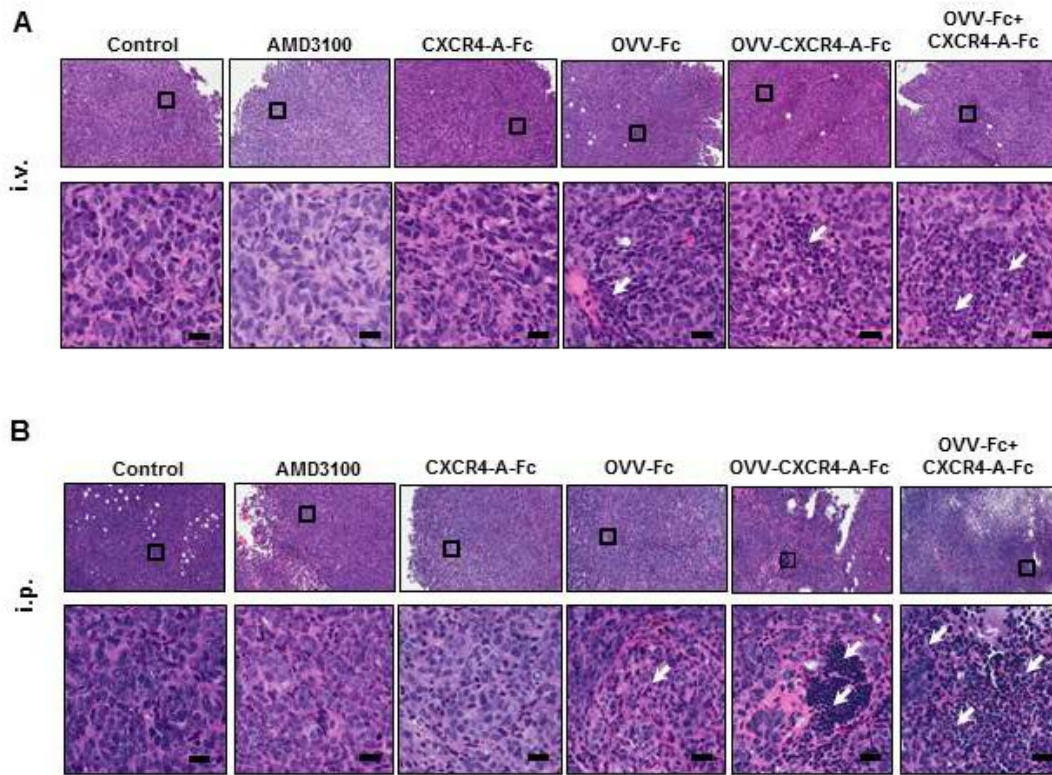
**Figure 4.** H&E staining of paraffin sections of omental tumors from mice treated with oncolytic virotherapy or CXCR4 antagonists delivered alone or in combination. C57BL/6 mice ($n = 5$) were challenged orthotopically with ID8-T tumor cells ($5 \times 10^5$ cells). The tumor-bearing mice were treated with AMD3100 or CXCR4-A-Fc (5 mg/kg) antagonists, OVV-Fc, OVV-CXCR4-A-Fc ($10^8$ PFU), or a combination treatment of OVV-Fc and CXCR4-A-Fc injected i.v. (**A**) or i.p. (**B**) 10 days after tumor challenge. Control mice were treated with PBS. Thirty days after each treatment, omental tumors were removed fixed, paraffin embedded, and sectioned. Five-micrometer-thick sections were stained with H&E. (Scale bars, 25 μm). Arrows indicate infiltration of leukocytes in tumor tissues.

**Task 3:** *In vivo* pathogenicity of the CXCR4 antagonist delivered intravenously or intraperitoneally was determined by histology on formalin fixed tissues and a complete blood count (CBC) on peripheral blood.

**Progress:**

3a. *H&E staining of paraffin sections of formalin fixed tissues and CBCs in mice treated with oncolytic virotherapy or CXCR4 antagonists delivered alone or in combination (timeframe, during months 6-11): In vivo* pathogenicity of the CXCR4-A-Fc fusion protein delivered i.v. or i.p. in a form of oncolytic virotherapy with the CXCR4 antagonist-expressing vaccinia virus or injected as a soluble protein was determined by examination of formalin-fixed tissues of spleen, inguinal lymph node, BM, liver, kidney, and heart embedded in paraffin and stained with H&E. The analysis performed 30 days post-treatment revealed no organ damage after systemic or intraperitoneal deliveries of the virus alone or in combination with the soluble CXCR4 antagonists (Supplementary Figures 1 and 2, respectively), indicating no long-term pathogenic effect.

In parallel experiments, we have analyzed changes in the CBC profile in tumor-bearing mice to evaluate the off-target effect of the oncolytic virotherapy delivered alone or in combination with the CXCR4 antagonists on the BM pathology. The analysis was performed on heparinized peripheral blood samples collected from mice before (as a baseline level) and on days 8, 15, and 30 after each treatment. We conducted the study only after a systemic delivery of the virus and the CXCR4 antagonist because of higher concentrations of the CXCR4-A-Fc protein in sera compared to those measured after i.p. injection ($P = 0.015$) and accumulation of the antagonist in

several organs, in contrast to only background levels measured after the i.p. treatment. The results of the CBC analyses depicted in Fig. 5A and B showed, except for small fluctuations, no changes in numbers of red blood cells and platelets during the 30-day post-treatment period. We detected increases in leukocyte counts on days 8 after each treatment, which persisted for approximately one week before returning to the baseline numbers. The soluble CXCR4-A-Fc fusion protein and AMD3100 antagonist increased numbers of leukocytes by ~10% on days 8 and 15. The changes in white blood cell (WBC) counts were more pronounced after virotherapy treatments and were on average 30% higher compared to the baseline, which is in line with the induction of inflammatory responses. Thus, consistent with the histologic evaluation, no long-term toxicities were observed after treatments with the oncolytic vaccinia virus alone or in combination with the CXCR4-A-Fc antagonist.
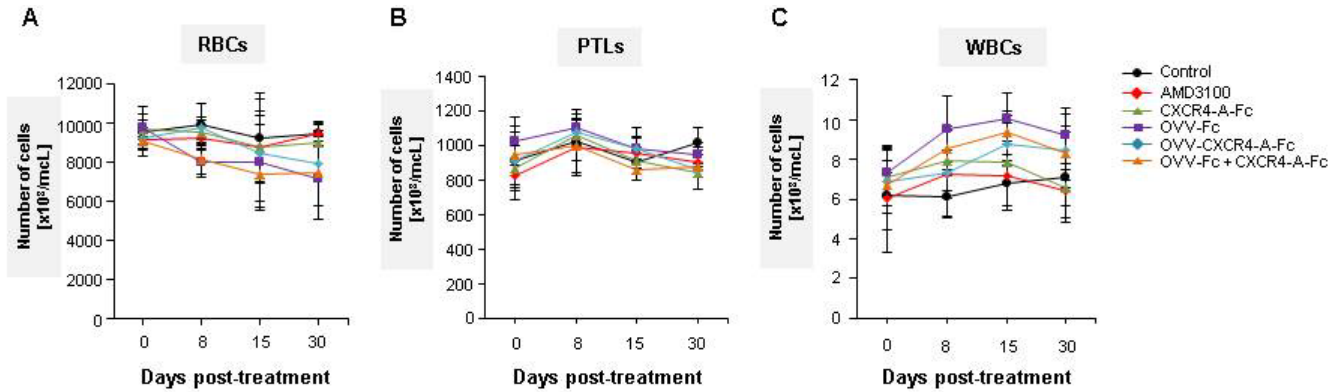


**Figure 5.** The effect of oncolytic vaccinia virus delivered alone or in combination with soluble CXCR4 antagonists on numbers of red blood cells (RBCs), platelets (PLTs) and WBCs. Mice ($n = 5$) were bled from the retro-orbital sinus to obtain complete CBCs before treatment and on days 8, 15 and 30 after treatment initiation. The numbers of RBCs (**A**), PLTs (**B**) and WBCs (**C**) in the heparinized blood samples were determined using IDEXX ProCyte Dx Hematology analyzer (IDEXX Laboratories, Inc., Westbrook, ME).

**Task 4:** Tumor infiltrating myeloid and CD8$^+$ T cells were characterized by flow cytometry analysis and the induction of Wilm's tumor antigen 1 (WT1)-specific antibodies were measured in sera of ID8-T tumor-bearing mice by ELISA.

**Progress:**

4a. *The induction of antitumor immune responses (timeframe, during months 1-12):* Because the intratumoral injection of the CXCR4-A-Fc antagonist in combination with vaccinia virus provided the highest antitumor efficacy with the lowest off-target effect, we next investigated the treatment-induced inflammatory responses associated with the generation of spontaneous antitumor immunity by analyzing intratumoral infiltration of dendritic cells (DCs), tumor-associated macrophages (TAMs), and a differentiation status of CD8$^+$ T lymphocytes along with their specificity for a clinically relevant WT1 antigen expressed by ID8-R cells (1, 4). The analysis of phagocytes was performed by immunofluorescence staining of single-cell suspensions prepared from ascites or peritoneal fluids of tumor-bearing mice using mAbs specific for CD11c$^+$ and CD86$^+$ DCs, CD11b$^+$ and F4/80$^+$ TAMs with M1 (expressing IL-12 cytokine) or M2 (expressing IL-10 cytokine) phenotype. The differentiation status of CD8$^+$ T lymphocytes and their WT1-specificity in the same tumor tissues as well as spleen and lymph nodes was investigated using mAbs specific for CD62L and CD44 antigens and tetramer specific for WT1$_{126-134}$ epitope (RMFPNAPYL) with H2-D$^b$-binding motif. In parallel, sera specimens (dilution of 1:100) were analyzed by ELISA for the presence of Abs to WT1 antigen as a surrogate marker of the treatment-induced antitumor immune responses.

The flow cytometry analysis performed on day 8 after completion of each treatment revealed ~10-fold higher numbers of tumor-infiltrating DCs after injection of OVV-CXCR4-A-Fc or the combination of OVV-Fc and soluble CXCR4-A-Fc protein compared to control mice (Fig. 6). On the other hand, injections of the soluble

CXCR4 antagonists increased numbers of tumor-infiltrating DCs by 2- to 3-fold. The changes were associated with higher percentages of IL-12 cytokine-expressing TAMs and lower numbers of IL-10 cytokine-expressing immunosuppressive macrophages.

| Treatment | %CD11c+CD86+ | %CD11b+F4/80+ | | |
|---|---|---|---|---|
| | | Total | IL-12+ | IL-10+ |
| Control | 0.7 ± 0.3 | 17.9 ± 3.5 | 3.4 ± 2.1 | 10.1 ± 3.8 |
| AMD-3100 | 1.3 ± 0.5 | 21.7 ± 4.0 | 8.5 ± 2.5 | 11.7 ± 4.1 |
| CXCR4 | 3.8 ± 0.9 | 26.0 ± 5.0 | 13.8 ± 4.1 | 9.1 ± 0.9 |
| OVV-Fc | 4.2 ± 1.0 | 31.7 ± 9.1 | 23.1 ± 5.4 | 2.2 ± 0.4 |
| OVV-CXCR4-A-Fc | 7.7 ± 1.2 | 36.1 ± 9.5 | 28.2 ± 4.5 | 4.8 ± 0.8 |
| OVV-Fc + CXCR4 | 8.8 ± 1.8 | 38.2 ±11.5 | 31.7 ± 6.9 | 2.0 ± 0.4 |

**Figure 6.** Analysis of DCs and TAMs in ascites-derived ID8-T tumors or peritoneal washes after oncolytic virotherapy and CXCR4 antagonists delivered alone or in combination. The percentages of CD11c+CD86+ DCs and CD11b+F4/80+ macrophages were analyzed on day 8 after treatment by staining with specific mAbs and flow cytometry. Background staining was assessed using isotype control antibodies. Results are presented as mean ± SD of five mice per group.

The induction of inflammatory responses in ID8-T tumor-bearing mice by the virus alone or in combination with the CXCR4 antagonist was also reflected in changes in the size and breadth of tumor-infiltrating CD8+ memory pools of T cells that are needed to enhance the efficacy of oncolytic virotherapy. The flow cytometry analysis revealed the highest percentages of CD44+CD62L- effector memory CD8+ T cells in tumors of mice treated with the virus alone or in combination with the CXCR4-A-Fc protein, which was increased over 10-fold compared to numbers measured in control mice or animals treated with the soluble CXCR4 antagonist (Fig. 7A). The percentages of CD44-CD62L+ naïve and double-positive central memory cells were low in all groups of tumor-bearing mice, indicating massive infiltration of effector CD8+ T cells to the sides of virally-induced inflammation in tumor tissues.
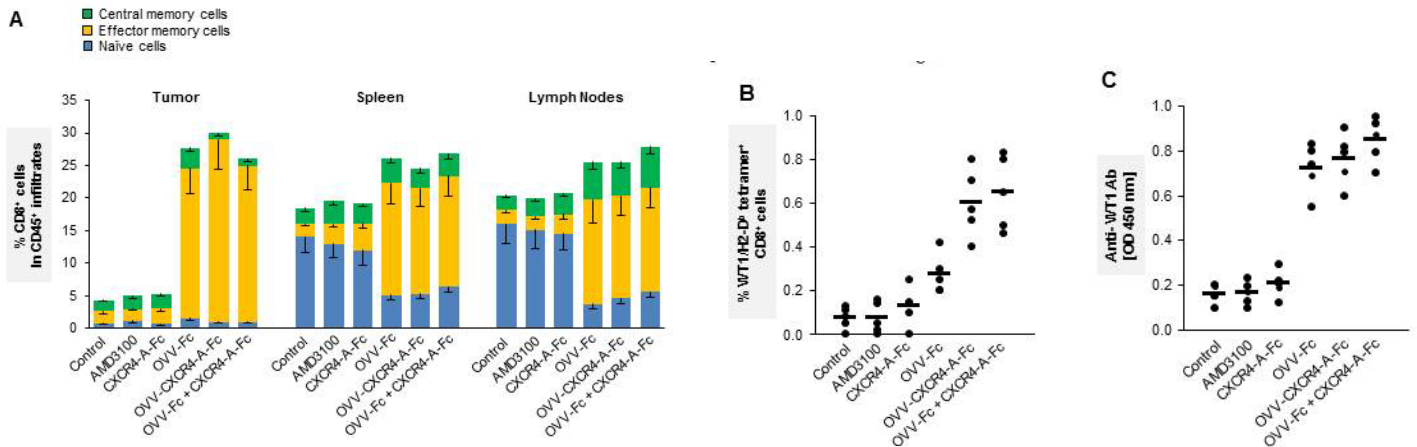


**Figure 7.** The effect of CXCR4 antagonists and oncolytic vaccinia virus on the phenotype of CD8+ T cells and the generation of WT1-specific immune responses. (**A**) Phenotypic analyses of CD8+ immune infiltrates in ascites-derived tumors or peritoneal washes as well as splenocytes and lymphocytes obtained from axillary, brachial, and inguinal lymph nodes were performed on CD45-gated cells with mAbs specific for CD44 and CD62L antigens on day 8 after treatment. Background staining was assessed using isotype control antibodies. Results are presented as mean ± SD of five mice per group. (**B**). The percentage of WT1$_{126-134}$ tetramer-specific CD8+ T cells in ascites-derived tumors or peritoneal washes of the same groups of ID8-T tumor-bearing mice was determined by staining with anti-CD8-PECy5 mAb and PE-labeled H-2D$^b$-restricted WT1$_{126-134}$ tetramer. Background staining was assessed using isotype control antibodies. (**C**) To measure the levels of WT1-specific Abs, sera (dilution 1:100) were analyzed by ELISA on wells coated with 3 μg/ml WT1 peptide (AAPPTec, Louisville, KY). Each point denotes an individual mouse.

The virally-induced effector CD8$^+$ T cell populations were also elevated by ~5-fold in spleen and lymph nodes of mice with increased infiltration of DCs and M1 macrophages in tumor tissues, which contrasted with twofold lower numbers of CD44$^-$CD62L$^+$ naïve T cells. On the other hand, the percentages of double-positive central memory CD8$^+$ T cells were low and did not significantly differ among different groups of tumor-bearing mice. The increased numbers of CD8$^+$ effector T cells in the tumor were also associated with the presence of anti-WT1 immune responses. As shown in Fig. 7B, less than 1% of CD8$^+$ T cells reacted with the H-2D$^b$-restricted WT112$_{6-134}$ tetramer in virotherapy-treated tumors, and sera of these mice also exhibited detectable anti-WT1 antibody responses (Fig. 7C). The challenge for the future investigation is to design strategies for boosting the oncolytic virotherapy-induced antitumor immune responses and more effective inhibition of ovarian tumor growth using prime-boost approaches with DC cancer vaccines and adoptively-transferred T cells.

**What was accomplished under these goals?**

**Key Research Accomplishments**: We have completed major elements of Aim 1.

1) We have demonstrated that the antitumor efficacy of intraperitoneal delivery of oncolytic virotherapy or the CXCR4 antagonist was higher than that of the intravenous injection.

2) The highest antitumor efficacy against malignant ID8-T ovarian tumor was achieved after intraperitoneal delivery of oncolytic vaccinia virus and soluble CXCR4-A-fusion protein.

3) Inhibition of ID8-T ovarian tumor growth was dependent on the induction of inflammatory responses and accumulation of the soluble CXCR4-A-Fc protein in tumor tissues.

4) Concentrations of the soluble CXCR4-A-Fc antagonist in sera, spleen, liver, BM, and lymph node tissues were higher after systemic than intraperitoneal delivery. In contrast, higher levels of the antagonist in tumor tissues compared to sera and other organs were measured after intraperitoneal delivery.

5) The treatment regiments of the oncolytic vaccinia virus ($10^8$ PFU) delivered intravenously or intraperitoneally alone or in combination with the soluble CXCR4-A-Fc antagonist (5 mg/kg) did not cause systemic toxicity.

**Conclusions**: The biology-driven rational design of novel immunotherapies will be critical for the development of a low side-effect cancer treatment that is based on the inhibition of the CXCL12/CXCR4 signaling axis which affects multiple aspects of ovarian tumor growth. The findings of this study have established that intraperitoneal treatment with oncolytic vaccinia virus in combination with the CXCR4-A-Fc antagonist is more beneficial as an adjuvant for cancer immunotherapy than systemic delivery of these agents. The induction of strong inflammatory responses and high levels of the CXCR4 antagonist in the tumor appear to be responsible for the intratumoral infiltration of effector CD8$^+$ T cells that contribute to the overall antitumor efficacy of the treatment. The challenge for our future investigation is to determine whether this combination can both engage the patient's immune system to promote *de novo* antitumor immune responses as well as augment vaccine-induced antitumor immunity.

**What opportunities for training and professional development has the project provided?**
Nothing to Report.

**How were these results disseminated to communities of interest?**
Nothing to Report.

**What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we will focus on understanding the mechanisms of antitumor activity of the CXCR4-A-Fc antagonist delivered in combination with oncolytic vaccinia virus and perform experiments outlined in the original SOW.

1. We will analyze the mechanisms of increased tumor cell death by the combined treatment in tumor-bearing mice focusing on the effect of the CXCR4-A-Fc antagonist on functions of virally-induced innate immune responses. We hypothesize that the combined treatment of oncolytic virotherapy and the CXCR4-A-Fc antagonist is able to increase tumor cell death based on the ability of the CXCR4-A-Fc antagonist to induce apoptosis in ID8-T cells (5) and mediate antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (1, 2). All these mechanisms may contribute to increases in antitumor efficacy in mice with high intratumoral levels of the CXCR4-A-Fc antagonist and inflammatory responses.

2. Because the accumulation of the injected CXCR4-A-Fc antagonist in the peritoneal cavity of tumor-bearing mice was at least 2-fold higher compared to that released from the virally-infected tumor cells, we are analyzing inhibition of tumor growth by the armed OVV-CXCR4-A-Fc virus delivered twice in a lower titer ($5 \times 10^7$ PFU) within a 7-day period. This experiment will determine whether a sustained concentration of the soluble CXCR4-A-Fc antagonist in the tumor microenvironment after two injections, opposite to a single peak monitored 4 days after single viral delivery, will improve efficacy of the armed virus. Establishing the most efficacious approach of the CXCR4 antagonist delivery may have translational relevance as injections of the armed vaccinia virus represent a less complicated approach than combining two separate treatment modalities.

3. As outlined in the original SOW, we will evaluate the effect of the most efficacious virotherapy treatment on the antitumor efficacy of dendritic cell vaccines (Aim 2) and adoptively-transferred T cells (Aim 3) as well as explore the mechanisms associated with the induction of protective antitumor immune responses.

4. We will validate the key findings obtained in the ID8-T-challenged C57BL/6 mice using C57BL/6 TgMISIIR-TAg-Low transgenic mice that serve as immunocompetent syngeneic allograft recipients for ovarian MOVCAR cell line (6). Based on distinct characteristics of the MOVCAR cell line and its flexibility in manipulation of gene expression, this model represents an amenable system to study ovarian tumor biology and to evaluate the efficacy of novel therapeutic strategies. This transgenic model has been provided to us by Dr. Denise C. Connolly at Fox Chase Cancer Center, Philadelphia, PA.

All further studies described in the original application remain essentially unchanged. Based on the high antitumor efficacy with no systemic toxicity of the combined oncolytic virotherapy and the CXCR4-A-Fc antagonist treatment, we believe that our observation has a strong likelihood of success in providing a targeted therapy against aggressive ovarian cancer with the longer progression-free survival and improved quality of life.

**4.      IMPACT**
**What was the impact on the development of the principal discipline(s) of the project?**
Nothing to Report.

**What was the impact on other disciplines?**
Nothing to Report.

**What was the impact on technology transfer?**
Nothing to report.

**What was the impact on society beyond science and technology?**
Nothing to Report.


**5.     CHANGES/PROBLEMS:**
Nothing to report.


**6.     PRODUCTS:**
Nothing to Report.


**7.     PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
**What individuals have worked on the project?**

Danuta Kozbor, Ph.D.
PI
Effort:  2.4 calendar months (no change)

Adekunle Odunsi, M.D., Ph.D.
Co-Investigator
Effort:  0.24 calendar months (no change)

Austin Miller, Ph.D.
Biostatistician
Effort:  0.36 calendar months (no change)

Marcin Komorowski, M.Sc.
Research Affiliate
Effort:  9.6 calendar months (no change)

Agnieszka Kolakowska, M.Sc.
Research Affiliate
Effort:  8.0 calendar months (no change)

Robert A.J. McGray, Ph.D.
Post-doctoral Associate
Effort:  1.2 calendar months (no change)


**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
Nothing to Report.

**What other organizations were involved as partners?**
Nothing to Report.

**8.     SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:** Not applicable

**QUAD CHARTS:** Not applicable

**9.     APPENDICES:**

- Updated Curriculum Vitae

- Supplementary Information: Supplementary Figures 1 and 2

**REFERENCES**

1.  Gil, M., Komorowski, M.P., Seshadri, M., Rokita, H., McGray, A.J., Opyrchal, M., Odunsi, K.O., and Kozbor, D. 2014. CXCL12/CXCR4 blockade by oncolytic virotherapy inhibits ovarian cancer growth by decreasing immunosuppression and targeting cancer-initiating cells. *J Immunol* 193:5327-5337.
2.  Gil, M., Seshadri, M., Komorowski, M.P., Abrams, S.I., and Kozbor, D. 2013. Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases. *Proc Natl Acad Sci U S A* 110:E1291-1300.
3.  Zhu, J., Martinez, J., Huang, X., and Yang, Y. 2007. Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN-beta. *Blood* 109:619-625.
4.  Hylander, B., Repasky, E., Shrikant, P., Intengan, M., Beck, A., Driscoll, D., Singhal, P., Lele, S., and Odunsi, K. 2006. Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. *Gynecol Oncol* 101:12-17.
5.  Komorowski, M.P., McGray, A.R., Kolakowska, A., Eng, K., Gil, M., Opyrchal, M., Litwinska, B., Nemeth, M.J., Odunsi, K.O., and Kozbor, D. 2016. Reprogramming antitumor immunity against chemoresistant ovarian cancer by a CXCR4 antagonist-armed viral oncotherapy. *Mol Ther Oncolytics* 3:16034-16048.
6.  Quinn, B.A., Xiao, F., Bickel, L., Martin, L., Hua, X., Klein-Szanto, A., and Connolly, D.C. 2010. Development of a syngeneic mouse model of epithelial ovarian cancer. *J Ovarian Res* 3:24-41.

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Danuta Kozbor

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE:  Associate Professor and Associate Member

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | Completion Date MM/YYYY | FIELD OF STUDY |
|---|---|---|---|
| VII General Ed. Lyceum, Poland | B.Sc | 06/1971 | Biology |
| Jagiellonian University, Krakow, Poland | M.Sc | 06/1976 | Molecular Biology |
| Karolinska Institute, Stockholm, Sweden | | 12/1978 | Tumor Biology |
| Queen's University, Kingston, Ontario, Canada | Ph.D. | 10/1982 | Microbiol/Immunol |

## A.  Personal Statement

The overall goal of our research is to elucidate molecular and cellular mechanisms of antitumor activities of immuno-oncolytic viral vectors against primary and metastatic ovarian tumors. We design novel approaches to enhance the antitumor efficacy of oncolytic virotherapy by expressing therapeutic genes and augmenting spread of the virus within tumors in preclinical studies in mice. Specifically, we have evaluated the effect of selective disruption of tumor vasculature by photodynamic therapy (PDT) on the therapeutic activity of systemically administered thymidine kinase (*TK*)- and vaccinia growth factor (*VGF*)-deleted oncolytic vaccinia virus.  Using highly metastatic variants of ovarian tumors, we investigate methods to reprogram antitumor immune responses by a CXCR4 antagonist-armed oncolytic vaccinia virus.  We anticipate that the oncotherapy-mediated changes in the tumor microenvironment will modulate the interaction between malignant and stromal cells and facilitate induction of protective antitumor immune responses. These studies, if successful, have the potential to be translated into the clinic though collaboration with our clinical investigators and will help in exploring the mechanistic underpinnings of tumor-immune system interactions.

As a postdoctoral fellow at the Wistar Institute in Philadelphia, PA, I gained experience in molecular genetics during the research on the mechanism of oncogene activation in human tumors by chromosomal translocation.  Subsequently, as an independent investigator, I expanded my research to virology by analyzing the effect of perinatal HIV infection of disease progression, and induction of HIV-specific immune responses using a recombinant vaccinia virus as a vector for vaccine delivery.  As PI or Co-Investigator on several previous NIH- and institute-funded grants, I laid the background for the proposed research by developing oncolytic vaccinia viruses expressing mimotopes of tumor associated antigens and by targeting the antigens to the activating Fc gamma receptor on dendritic cells.  In addition, I successfully administered the projects (e.g. staffing and budget), collaborated with other researchers, and produced several peer-reviewed publications from each study. I am aware of the importance of frequent communication among project members and constructing a realistic research plan, timeline and budget.

## B.  Positions and Honors

**Positions and Employment**

| | |
|---|---|
| 1982-1985 | Postdoctoral Fellow, National Cancer Institute of Canada, Wistar Institute, Philadelphia, PA |
| 1985-1989 | Associate Scientist, The Wistar Institute, Philadelphia, PA |
| 1989-1991 | Assistant Professor, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA |
| 1991-1996 | Assistant Professor, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA |
| 1996-1998 | Associate Professor, Allegheny University of the Health Sciences, Philadelphia, PA |
| 1998-1999 | Associate Professor, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA |
| 1999-2002 | Professor, Center for Neurovirology and Cancer Biology, Temple University, Philadelphia, PA |
| 2002-present | Associate Professor and Associate Member, Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY |

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## Other Professional Activities and Honors

| | |
|---|---|
| 1980-1982 | National Cancer Institute of Canada Studentship |
| 1982-1986 | National Cancer Institute of Canada Fellowship |
| 1986-1987 | Consultant on the Hybridoma Technology, World Health Organization (WHO Regional Office for Southeast Asia, New Delhi, India) |
| 1986-1988 | Consultant on the Hybridoma Technology, Dow Chemicals, Midland, MI |
| 1988-1989 | Consultant on the Hybridoma Technology, DuPont Company, Wilmington, DE |
| 1994-1999 | Ph.D. Thesis Advisory Committee, University of Pennsylvania, Philadelphia, PA |
| 1996-1999 | Clinical Trials in Zambia: "A phase II evaluation of curdlan sulfate bolus infusion alone or in combination with quinine in patients infected with malaria". |
| 1995-2002 | Investigator, Philadelphia Pediatric AIDS Clinical Trials Unit |
| 1999-2002 | Investigator, Penn Center for AIDS Research, Philadelphia, PA |
| 1999-2006 | Editorial Board Member, Clinical & Diagnostic Laboratory Immunology |
| 1999-2007 | Reviewer, NIH/NIAID Special Emphasis Review Panel on HIV Vaccine Research & Design NIH-NIAID-RFA-05001 Leadership for HIV/AIDS Clinical Trials Networks: Vaccines. |
| 2000 | Visiting Professor, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan2006 |
| 2006 | Patent: March 14, 2006; "β-Glucans encapsulated in liposomes" (US patent 7,011.845). |
| 2004-2013 | Chair, Institute Biosafety Committee, Roswell Park Cancer Institute, Buffalo, NY |
| 2012 | *Ad Hoc* Reviewer; NIH, Cancer Immunopathology and Immunotherapy Study Section |

## C. Contributions to Science

### 1. Cancer Immunotherapy and Vaccines

A major challenge for inducing antitumor immune responses with native or modified tumor/self-Ags in tumor-bearing hosts relates to achieving efficient uptake and processing by dendritic cells (DCs) to activate immune effector cells and limit the generation of immunosuppressive network in the tumor microenvironment. We have demonstrated that immunization of adoptively transferred T cells in tumor-bearing mice with a CD166 cross-reactive mimotope 47-LDA, expressed in the context of the activating Fc fusion protein, induced higher levels of antitumor immune responses and protection than the 47-LDA polypeptide-DC vaccine. The antitumor efficacy of the therapeutic 47-LDA-Fc-DC vaccine was comparable to that achieved by an oncolytic vaccinia virus (OVV) expressing the 47-LDA-Fc fusion protein, paving the way for testing novel anticancer treatments.

Because the CXCR4 receptor for the CXCL12 chemokine is one of the key stimuli involved in signaling interactions between tumor cells and their microenvironment, we have also investigated whether inhibition of this pathway by oncolytic viruses expressing the CXCR4 antagonist increases efficacy over that mediated by oncolysis alone. We are unique in demonstrating that targeting CXCR4 signaling through an oncolytic vaccinia virus yields a significant therapeutic impact against primary and metastatic breast and ovarian cancers. I served as a senior investigator in all of these studies.

Gil, M., Bieniasz, M.,  Wierzbicki, A., Bambach, B., Rokita, H., and Kozbor, D.  Targeting a Mimotope Vaccine to Activating Fc gamma Receptors Empower Dendritic Cells to Prime Specific CD8+ T Cell Responses in Tumor-bearing Mice. J. Immunology, 2009, 183:6808-18, PMCID: PMC2805007.

Gil M., Seshadri M., Abrams S. I, Kozbor, D.  Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases.  PNAS, 2013, 110:E1291-E1300, PMCID: PMC3619300.

Gil, M., Komorowski, M., Seshadri, M., Rokita, H., McGray, A.J.R., Opyrchal, M., Odunsi, K., and Kozbor, D.  CXCL12/CXCR4 blockade by oncolytic virotherapy inhibits ovarian cancer growth by decreasing immunosuppression and targeting cancer initiating cells. J.Immunol, 2014, 193:5327-5337, PMCID: PMC4225176.

Komorowski, M., McGray, A.J.R., Kolakowska, A., Eng, K., Gil, M., Opyrchal, M., Litwinska, B., Nemeth, M.J., Odunsi, K.O., and Kozbor, D.  Reprograming antitumor immunity against chemoresistant ovarian cancer by a CXCR4 antagonist-armed viral oncotherapy.  Mol. Therapy-Oncolytics, 2016, 3:16034-16048.

## 2.   T Cell Activation during Perinatal HIV Infection

The purpose of these studies was to examine the changes in cytokine/chemokine expression and T cell activation during progression towards AIDS in infants born to HIV-infected mothers as well as adult HIV-infected patients.  We have demonstrated for the first time a protective role of chemokine against vertical HIV infection, association of early HIV-specific Th1 and CTL responses with slow disease progression, and expansion of $V\delta1^+$T lymphocytes during progression of HIV infection. I served as a senior investigator in all of these studies.

Hyjek, E., Lischner, H. W., Hyslop, T. Bartkowiak, J., Kubin, M., Trinchieri, G., and Kozbor, D.  Cytokine patterns during progression to AIDS in children with perinatal HIV-1 infection.  J. Immunol., 1995, 155:4060-4071.

Hyjek, E. M., Bartkowiak, J., Drozdz, R., Wasik, T. J., Jasinski, M., Lischner, H. W. and Kozbor, D.  Evidence for B cell-mediated activation of $V\delta1^+$ T lymphocytes during progression of HIV infection. J. Immunol. 1997, 158:464-474.

Wasik, T. J., Jagodzinski, P. P., Hyjek, E. M., Lischner, H. W., and Kozbor, D.  Diminished HIV-specific CTL activity associated with enhanced type 2 responses to HIV-specific peptides during perinatal HIV infection. J. Immunol. 1997, 158:6029-6036.

Wasik, T. J., Lischner, H. W., Jasinski, M., Bratosiewicz, J., Whiteman, V. E., Rutstein, R., Starr, S. E., Douglas, S., Kaufman, D., Sison, A. V., and Kozbor, D.  Protective role of nonlytic immune responses in vertical HIV transmission.  J. Immunol. 1999, 162:4355-4364.

## 3.   Development of HIV Vaccine

In addition to the contribution described above, with a team of collaborators, I was involved in the development of HIV vaccines by identifying immunogenic and conserved epitopes within the HIV Envelope and Gag/Pol antigen for induction of protective cellular responses.  I served as a senior investigator in all of these studies.

Kmieciak, D., Jasinski, M., Teppler, H., Pientka, J., Hsu, S. H., Takahashi, H., Okumura, K., Kaneko, Y., and Kozbor, D.  The effect of deletion of the V3 loop of gp120 on induction of cytotoxic T cell responses and HIV gp120-mediated pathogenesis.  J. Immunol. 160:5676-5683, 1998.

Kmieciak, D., Bednarek, I., Takiguchi. M., Bratosiewicz, J., Wierzbicki, A., Wasik, T., Teppler, H., Pientka, J., Hsu, S. H., and Kozbor, D.  The effect of epitope variation on the profile of CTL responses to the HIV envelope glycoprotein. International Immunol. 10:1789-1799, 1998.

Bolesta E., Gzyl, J., Wierzbicki, A., Kmieciak, D., Kowalczyk, A., Srinivasan, A., Kaneko, Y., and Kozbor, D. Clustered Epitopes within Gag-Pol Fusion Protein DNA Vaccine Enhance Immune Responses and Protection against Challenge with Recombinant Vaccinia Viruses Expressing HIV-1 Gag and Pol Antigens. Virology 332:467-479, 2005.

Bolesta, E., Kowalczyk, A., Wierzbicki, A., Eppolito, C., Shrikant, PA. and Kozbor. D. Increased Level and Longevity of Protective Immune Responses Induced by DNA Vaccine Expressing the HIV-1 Env Glycoprotein when Combined with IL-21 and IL-15 Gene Delivery. J. Immunology, 2006, 177: 177-191.

## 4.    Mechanisms of Oncogene Activation in Human Malignancies

I was involved in characterizing mechanisms of oncogene activation (abl and myc) by chromosomal translocation in leukemia and solid tumors.

Kozbor, D., Finan, J., Nowell, P.C., and Croce, C.M. The gene encoding the T4 antigen maps to human chromosome 12. J. Immunol., 136: 1141-1143, 1986.

Kozbor, D., Giallongo, A., Sierzega, M.E., Konopka, J.B., Witte, O.N., Showe, L.C., and Croce, C.M. Expression of a translocated c-abl gene in hybrids of mouse fibroblasts and chronic myelogenous leukemia cells. Nature, 319: 331-333, 1986.

Kozbor, D., Moretta, A., Messner, H.A., Moretta., L., and Croce, C.M. Tp44 molecules involved in antigen-independent T cell activation are expressed on human plasma cells. J. Immunol., 138: 4128-4132, 1987.

Kozbor, D., Burioni, R., ar-Rushdi, A., Zmijewski, C., and Croce, C.M. Expression of members of immunoglobulin gene family in somatic cell hybrids between human B-and T-cells. Proc. Natl. Acad. Sci. USA, 84: 4969-4973, 1987.

## 5.    Production of Human Monoclonal Antibodies

Development of the hybridoma technique has revolutionized treatments of autoimmunity and cancer. I have pioneered the EBV-hybridoma technology for production of human monoclonal antibodies.

Kozbor, D. and Roder, J.C. Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein-Barr virus technique. J. Immunol. 127: 1275-1280, 1981.

Kozbor, D., Lagarde, A.E., and Roder, J.C. Human hybridomas constructed with antigen specific EBV-transformed cell lines. Proc. Natl. Acad. Sci, USA 79: 6651-6655, 1982.

Kozbor, D. and Roder, J.C. In vitro stimulated lymphocytes as a source of human hybridomas. Eur. J. Immunol. 14: 23-27, 1983.

Kozbor, D., Tripputi, P., Roder, J.C., and Croce, C.M. A human hybrid myeloma for production of human monoclonal antibodies. J. Immunol. 133(6): 3001-3005, 1984.

**Complete List of Published Work in My Bibliography:**
http://www.ncbi.nlm.nih.gov/sites/myncbi/danuta.kozbor.1/bibliography/44160804/public/?sort=date&direction=ascending

# SUPPLENTARY INFORMATION

# CXCR4 Antagonist as an Adjuvant in Immunotherapy of Epithelial Ovarian Cancer

PI: Danuta Kozbor, Ph.D.
   Department of Immunology
   Roswell Park Cancer Institute
   Buffalo, NY 14263

**Supplementary Figure 1.** H&E staining of paraffin sections of spleen, inguinal lymph node, bone marrow, liver, kidney and heart after intravenous delivery of oncolytic vaccinia virus or CXCR4 antagonists alone or in combination.

**Supplementary Figure 2.** H&E staining of paraffin sections of spleen, inguinal lymph node, bone marrow, liver, kidney and heart after intraperitoneal delivery of oncolytic vaccinia virus or CXCR4 antagonists alone or in combination.
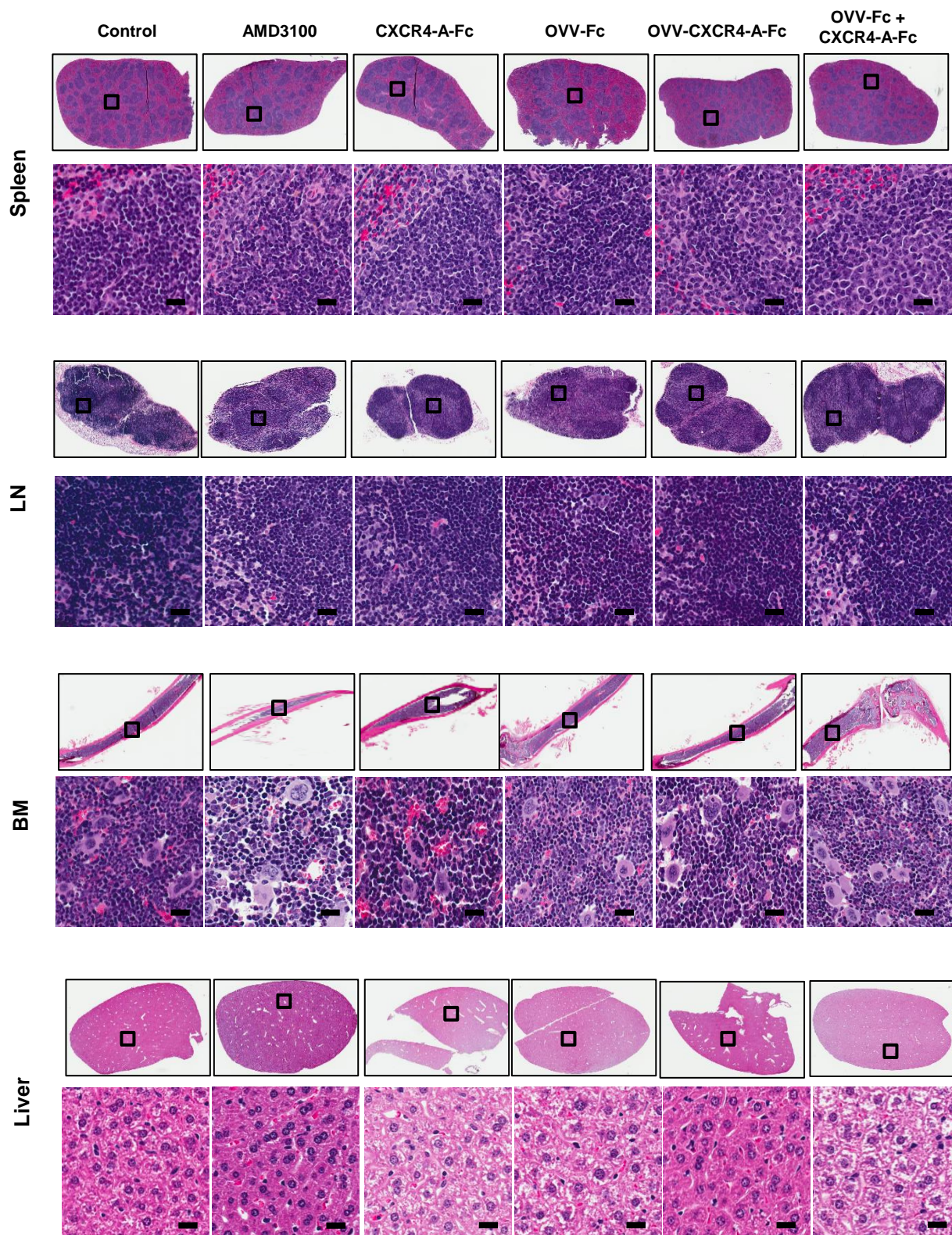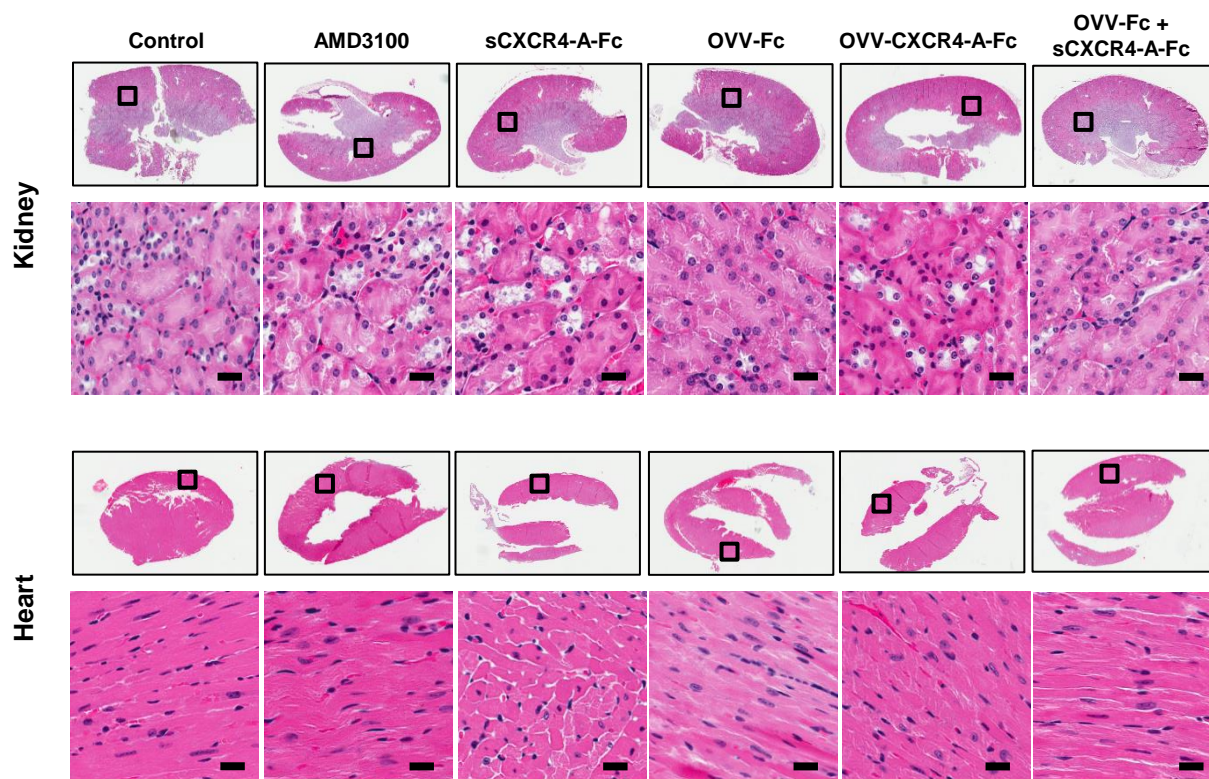
# Figure S1.  Intravenous delivery

# Figure S1.  Intravenous delivery



**Supplementary Figure 1. H&E staining of paraffin sections of spleen, inguinal lymph node (LN), bone marrow (BM), liver, kidney and heart after intravenous delivery of oncolytic vaccinia virus or CXCR4 antagonists alone or in combination.**  C57BL/6 mice ($n$ = 5) were challenged orthotopically with ID8-T tumor cells ($5 \times 10^5$ cells). The tumor-bearing mice were treated with AMD3100-a bicyclam or CXCR4-A-Fc (5 mg/kg) antagonists, OVV-Fc, OVV-CXCR4-A-Fc ($10^8$ PFU), or a combination treatment of OVV-Fc and CXCR4-A-Fc injected intravenously 10 days after tumor challenge.  Control mice were treated with PBS.  Thirty days after the treatment tissues from removed organ were fixed, paraffin embedded, and sectioned.  Five-micrometer-thick sections were stained with H&E. (Scale bars, 25 μm). Representative sections of the organs from one randomly selected mice are shown.
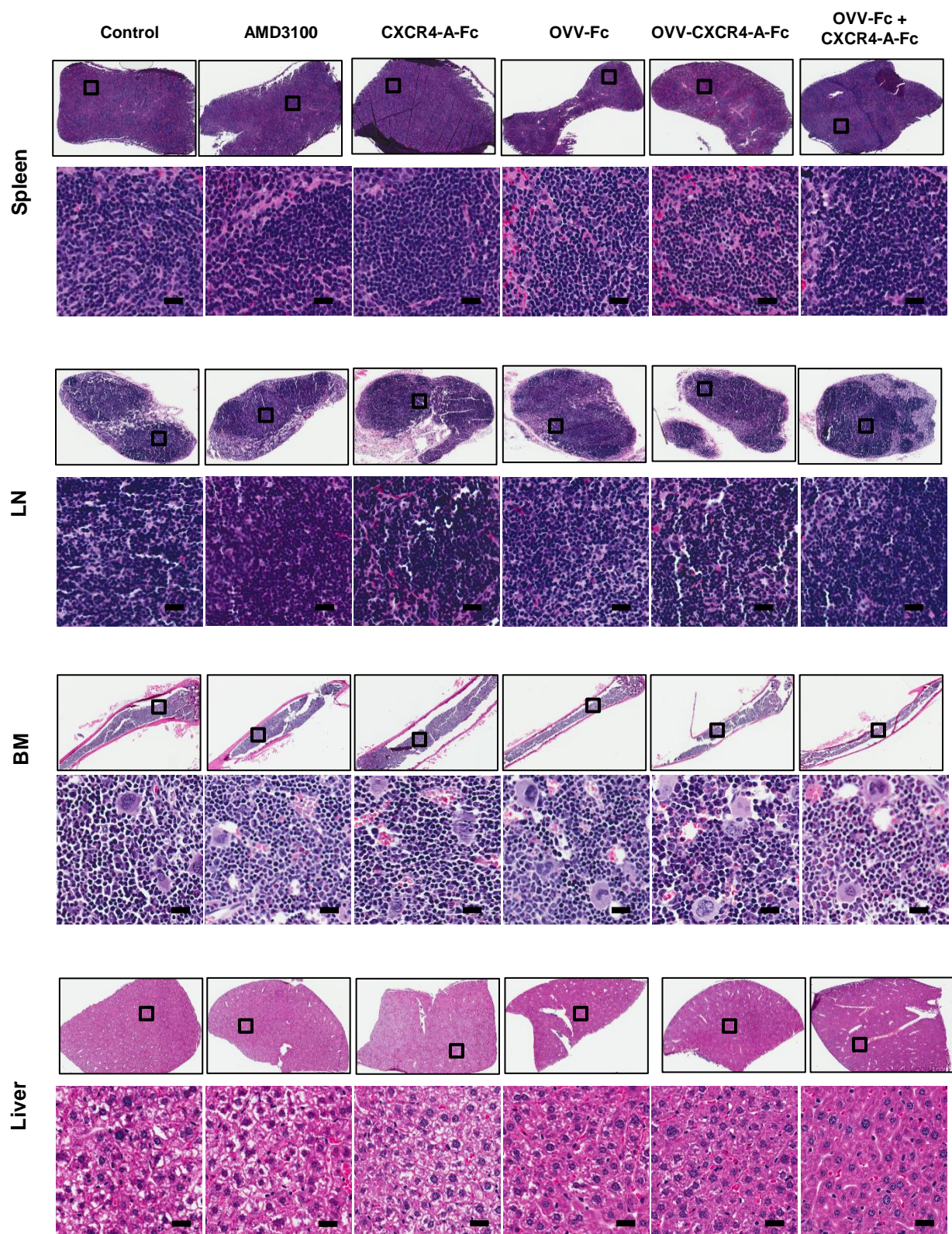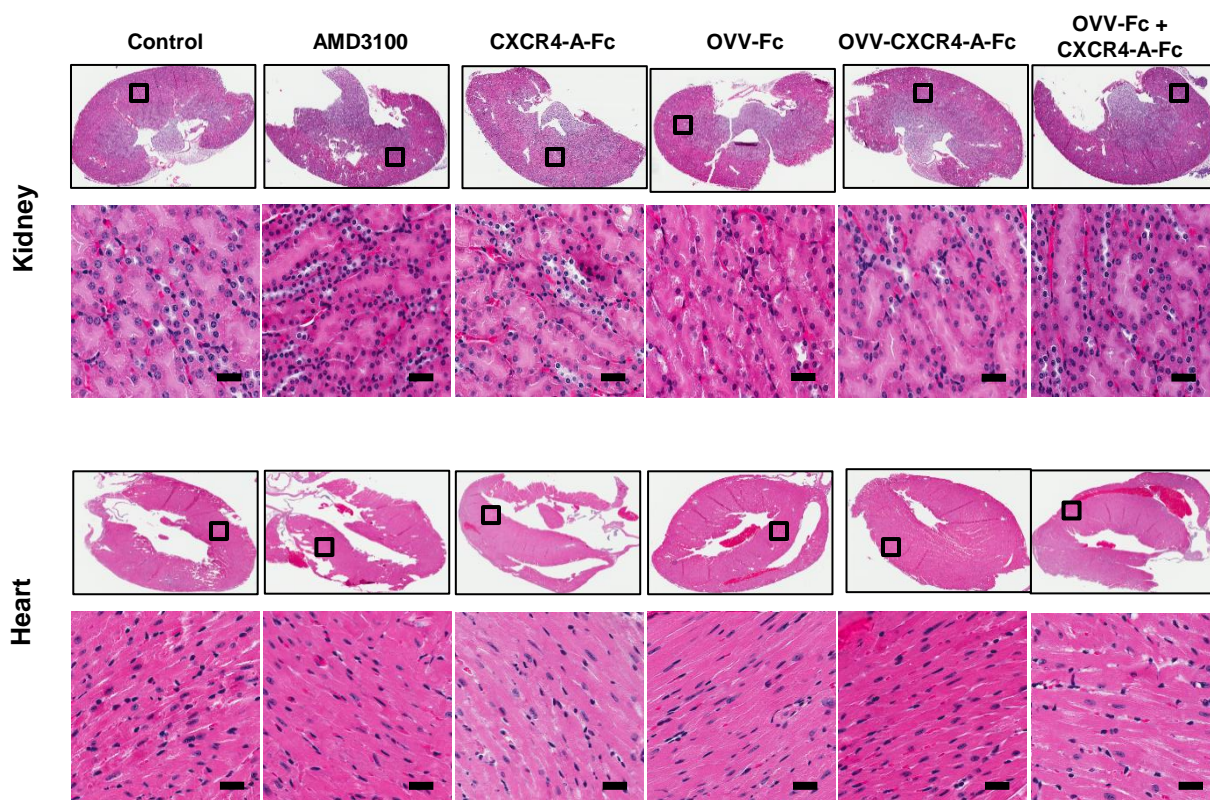
# Figure S2.   Intraperitoneal delivery

# Figure S2.  Intraperitoneal delivery



**Supplementary Figure 2. H&E staining of paraffin sections of spleen, inguinal lymph node (LN), BM, liver, kidney and heart after intraperitoneal delivery of oncolytic vaccinia virus or CXCR4 antagonists alone or in combination.** C57BL/6 mice ($n$ = 5) were challenged orthotopically with ID8-T tumor cells ($5\times10^5$ cells).  The tumor-bearing mice were treated with AMD3100-a bicyclam or CXCR4-A-Fc (5 mg/kg) antagonists, OVV-Fc, OVV-CXCR4-A-Fc ($10^8$ PFU), or a combination treatment of OVV-Fc and CXCR4-A-Fc injected intraperitoneally 10 days after tumor challenge.  Control mice were treated with PBS.  Thirty days after the treatment tissues from removed organ were fixed, paraffin embedded, and sectioned.  Five-micrometer-thick sections were stained with H&E. (Scale bars, 25 μm). Representative sections of the organs from randomly selected mice are shown.